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PRINCIPAL INVESTIGATOR: Colm Morrissey

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98195

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Colm Morrissey

Email: cmorriss@u.washington.edu

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14. ABSTRACT

A group of chromosomal translocations were recently discovered in prostate cancer that fuses the 5` region of TMPRSS2 (a serine protease) gene to the 3` region of ETS transcription factor genes (1). TMPRSS2 is an androgen responsive gene and contributes only its promoter region and usually a very short exon-1 (2, 3). This causes aberrant expression of an ETS transcription factor in response to androgen. The most common ETS member involved in prostate cancer chromosomal translocations is ERG but other members such as ETV1, ETV4 and ETV5 have been also observed (4, 5). The more aggressive prostate cancers often contain these translocations, thus potentially increasing their utility as both diagnostic and prognostic marker (6-8). Cell culture and transgenic animal models suggest that increased expression of ETS members, as a result of the chromosomal translocations, increase cell invasion without affecting the proliferative potential (9-11). However, in some xenograft models reducing expression of TMPRSS2-ERG protein slows down prostate cancer growth (12, 13). Therefore, ETS proteins emerge as potential novel targets for treatment of primary and/or metastatic disease in prostate cancer.

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Introduction

A group of chromosomal translocations were recently discovered in prostate cancer that fuses the 5` region of *TMPRSS2* (a serine protease) gene to the 3` region of *ETS* transcription factor genes (1). *TMPRSS2* is an androgen responsive gene and contributes only its promoter region and usually a very short exon-1 (2, 3). This causes aberrant expression of an ETS transcription factor in response to androgen. The most common *ETS* member involved in prostate cancer chromosomal translocations is *ERG* but other members such as *ETV1*, *ETV4* and *ETV5* have been also observed (4, 5). The more aggressive prostate cancers often contain these translocations, thus potentially increasing their utility as both diagnostic and prognostic marker (6-8). Cell culture and transgenic animal models suggest that increased expression of ETS members, as a result of the chromosomal translocations, increase cell invasion without affecting the proliferative potential (9-11). However, in some xenograft models reducing expression of TMPRSS2-ERG protein slows down prostate cancer growth (12, 13). Therefore, ETS proteins emerge as potential novel targets for treatment of primary and/or metastatic disease in prostate cancer.

We developed small molecule inhibitors that target protein products of chromosomal translocations containing ETS transcription factors (14). We further established that our lead compound, YK-4-279, directly binds to both ERG and ETV1 proteins (15). YK-4-279 inhibits ERG and ETV1 mediated transcriptional activity and subsequent cellular invasive phenotype of prostate cancer cell lines. These effects were only observed in prostate cancer cell lines containing ETS chromosomal translocations such as VCaP and LNCaP and absent in the PC3 prostate cancer cell line that does not contain any ETS chromosomal translocations. Expression of ERG in PC3 cells from an expression vector sensitized them to YK-4-279 and inhibiting ERG expression in VCaP resulted in resistance to YK-4-279 effect (15). Therefore, we hypothesize that targeting ETS family of transcription factors by small molecules will inhibit malignant phenotypes of human prostate cancer cells.

Keywords

Prostate Cancer, TMPRSS2-ERG, ERG, patient derived xenografts, YK-4-279, Tumor growth, Proliferation, ERG Inhibitor, and treatment resistance.

Overall Project Summary

The research involves laboratory studies utilizing xenograft models to test the hypothesis that targeting a member of the ETS transcription factor family with small molecules such as YK-4-279 may effectively treat prostate cancers. In year 1 Dr. Uren screened ETS transcription factors to test in animal models of prostate cancer at the University of Washington. Dr. Morrissey obtained all approvals to start the animal studies in year 2, and has consistently met and Skyped with Dr. Uren to discuss the fine details of the animal studies. Dr. Uren has screened a panel of derivatives and determined that none of the derivatives were more effective at inhibiting ERG than YK-4-279. Therefore we moved ahead with testing YK-4-279 in the xenograft models as proposed. Dr. Morrissey has tested YK-4-279 in 4 patient derived xenograft lines as proposed. One of the ERG positive xenograft lines LuCaP 23.1 responded to treatment (**Figure 1**), LuCaP 86.2 had a limited response (**Figure 2**), and LuCaP 35 did not respond to treatment (**Figure 3**). The ERG negative line LuCaP 96 as expected did not respond to treatment (**Figure 4**). There is limited toxicity due to weight loss in the animals in response to YK-4-279 treatment. This impacted the tumor volumes, serum PSA and survival outcomes for all xenograft lines. Further, after discussion and review of previously obtained and new data Dr. Uren has determined that the R-enantiomer of YK-4-279 had no effect on ERG activity and it had associated toxicity *in vitro* and *in vivo* (**Figure 5-7**). Therefore we have determined to use the S-enantiomer as the second derivative. Since the second derivative is the S-enantiomer, and we could not have determined that this would be the derivative of choice until after the first set of studies, we can only start into studying the second derivative in the four xenograft lines in subcutaneous studies now.

The molecular analysis of the tissues is currently underway. For all tissue acquired from the animals, half paraffin embedded and the remainder was flash frozen in OCT. The paraffin embedded tumors were sectioned and stained by hematoxylin and eosin. Then a tissue microarray was constructed. This TMA was stained for ki67 to assess proliferation (analysis of the data is currently underway) (**Figure 8**). All xenografts were also stained for ERG to ensure ERG positivity and to determine if the levels of ERG are altered in response to therapy (**Figure 9**). No significant differences in ERG expression were observed in the YK-4-279 treated animals. In addition we did a western analysis to determine if any changes in ERG expression could be observed, but there were no changes between YK-4-279 treated and untreated tumors (data not shown). To determine if ERG inhibition was occurring we were to analyze the expression of genes downstream of ERG. The literature identifies a number of genes that are expressed downstream of ERG in primary prostate cancer. To determine if a set of genes are expressed in prostate cancer metastases and the xenografts we did an independent study of primary prostate cancer, prostate cancer metastases and the LuCaP xenografts by gene expression analysis. We determined that the overlap between primary prostate cancer, metastases and the LuCaP xenografts was limited (**Figure 10**). Therefore we could not rely on the literature to look at markers of response. To overcome this we have isolated RNA from OCT blocks, the RNA quality and RNA integrity number was assessed and samples were used for RNASeq analysis. The RNASeq has been completed, but the data is currently being analyzed. Once we identify genes that are differentially expressed between the YK-4-279 and vehicle control groups as per the grant application we will purchase antibodies and stain the tissue microarrays to determine if the same changes are observed at the protein level.

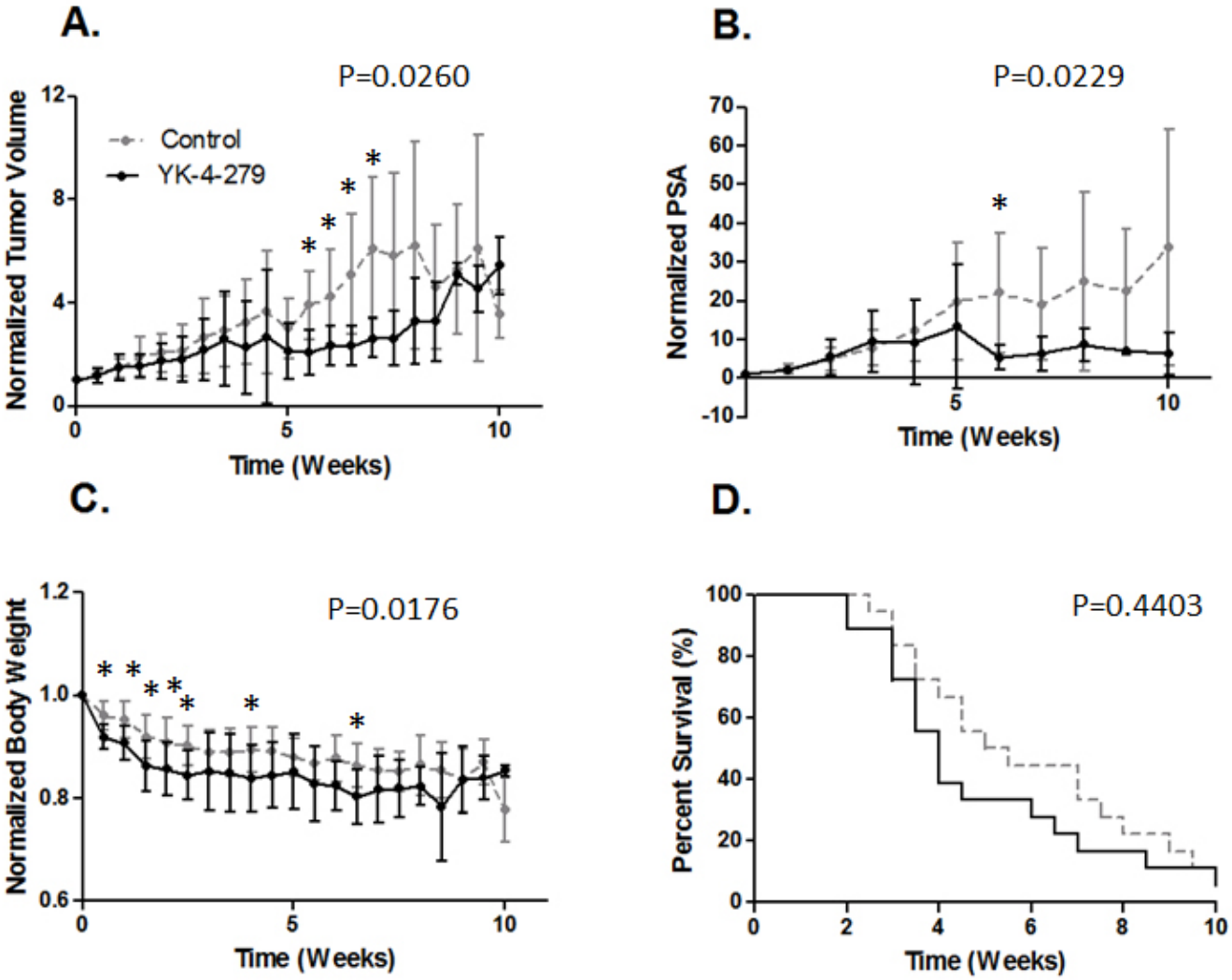


Figure 1. Response of animals bearing the ERG positive LuCaP 23.1 xenograft to YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG positive LuCaP 23.1 xenograft responded to ERG inhibition both at the tumor volume and serum PSA level. A significant change in body weight was observed in the animals treated with YK-4-279. No significant difference in survival was observed between the YK-4-279 and vehicle treated animals.

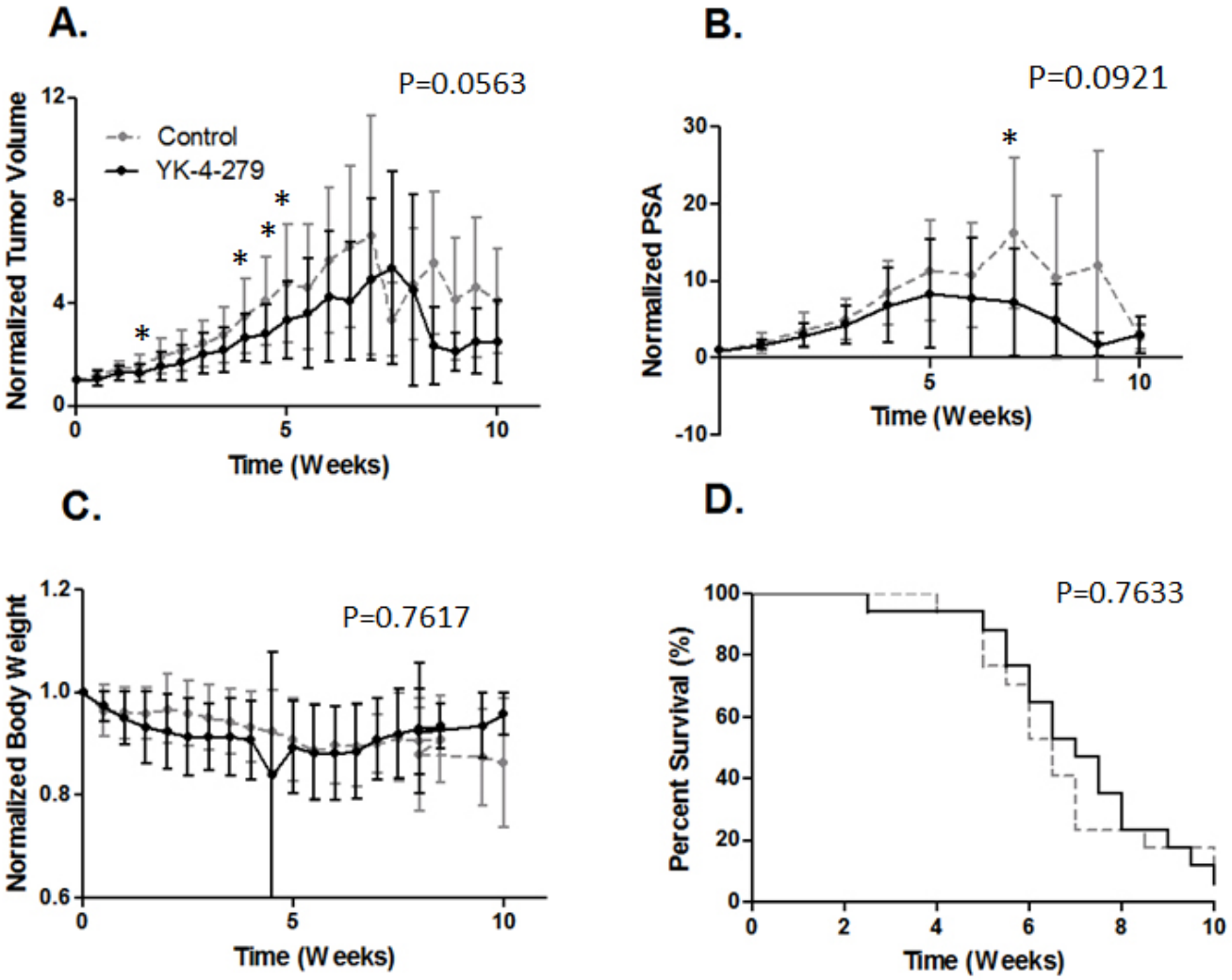


Figure 2. Response of animals bearing the ERG positive LuCaP 86.2 xenograft to YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG positive LuCaP 86.2 xenograft responded to ERG inhibition, (but was short of significance) both at the tumor volume and serum PSA level. No significant change in body weight was observed in the animals treated with YK-4-279. No significant difference in survival was observed between the YK-4-279 and vehicle treated animals.

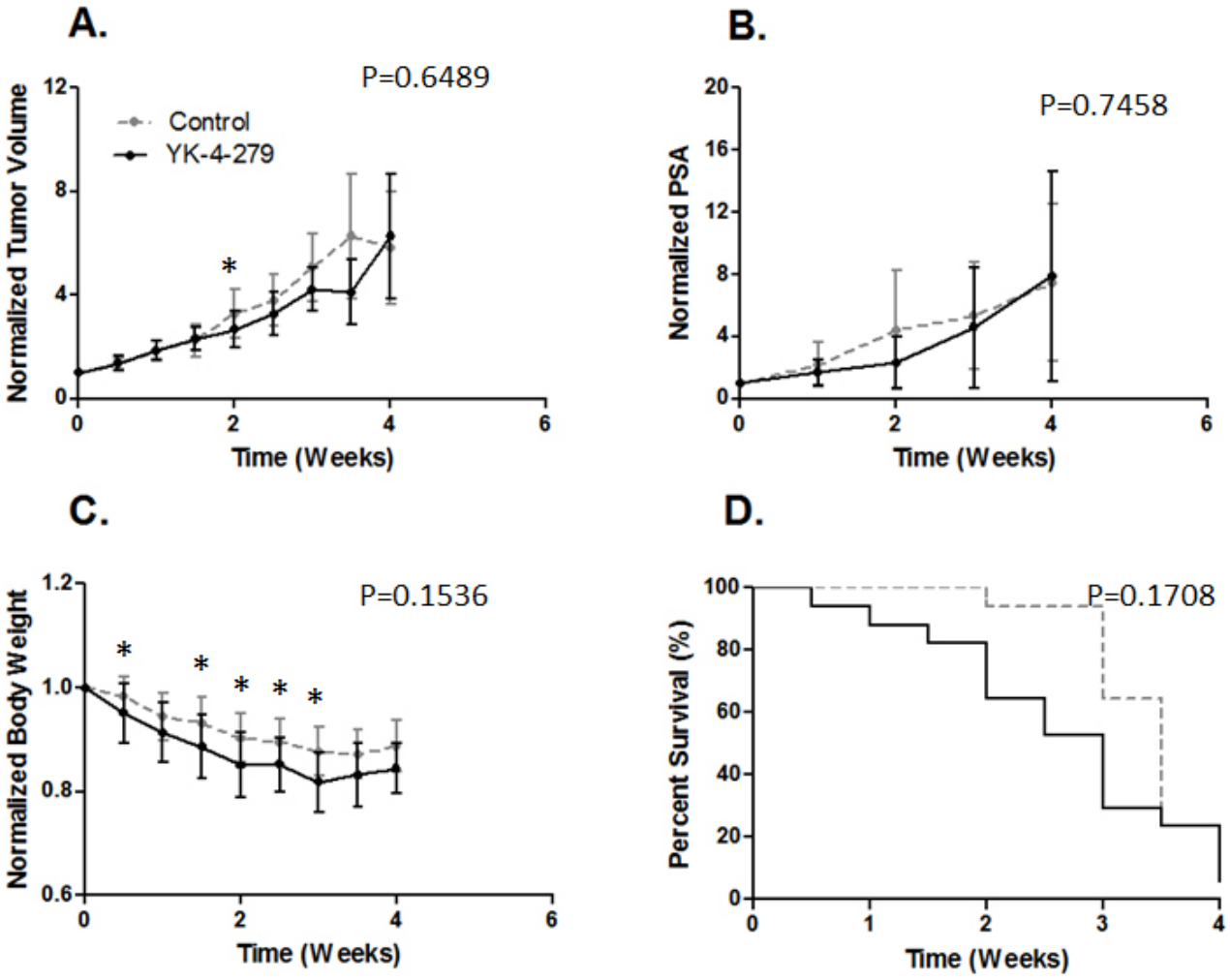


Figure 3. Response of animals bearing the ERG positive LuCaP 35 xenograft to YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG positive LuCaP 35 xenograft did not respond to ERG inhibition, both at the tumor volume and serum PSA level. No significant change in body weight was observed in the animals treated with YK-4-279. No significant difference in survival was observed between the YK-4-279 and vehicle treated animals.

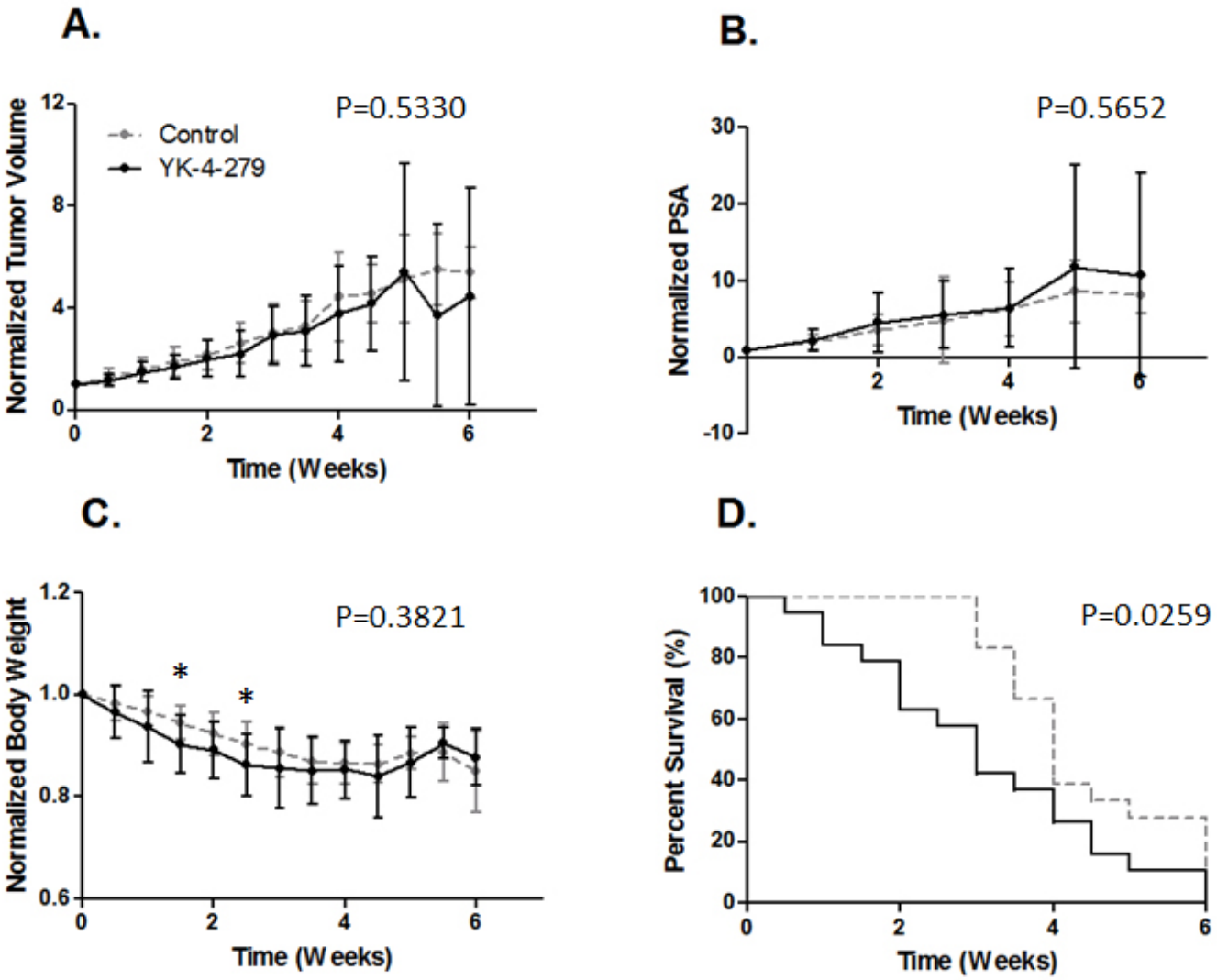


Figure 4. Response of animals bearing the ERG positive LuCaP 96 xenograft to YK-4-279. (A) Tumor volume, (B) Serum PSA, (C) Body weight, and (D) Survival. The ERG negative LuCaP 96 xenograft did not respond to ERG inhibition, both at the tumor volume and serum PSA level. No significant change in body weight was observed in the animals treated with YK-4-279. A significant difference in survival was observed between the YK-4-279 and vehicle treated animals.

Table 1: Cell growth effects of YK-4-279.

Cell Line	Histology	μM IC_{50} at 3 days (+/- SEM)					
		YK-4-279	Mean	(S)-YK-4-279	Mean	(R)-YK-4-279	Mean
TC32	ESFT (Type 1)	0.94 (0.14)	1.02 (0.89)	0.28 (0.06)	0.34 (0.09)	16.30 (4.83)	18.54 (4.95)
TC71	ESFT (Type 1)	1.83 (0.41)		0.16 (0.02)		20.86 (7.8)	
RDES	ESFT (Type 2)	1.03 (0.19)		0.87 (0.64)		12.71 (0.11)	
SKES	ESFT (Type 2)	0.33 (0.03)		0.18 (0.01)		21.01 (3.91)	
MMH-ES-1	ESFT (Type 2)	0.94 (0.13)		0.34 (0.08)		25.98 (4.03)	
STA-ET 7.2	ESFT (Type 2)	0.60 (0.04)		0.31 (0.01)		21.25 (3.49)	
A4573	ESFT (Type 3)	1.46 (0.31)		0.23 (0.08)		11.69 (6.56)	
PC3	prostate	4.95 (3.62)	8.88 (4.23)	3.79 (3.16)	6.86 (3.38)	>30 (0)	27.38 (1.67)
MCF7	breast	22.82 (7.19)		19.47 (10.53)		>30 (0)	
MDA-MB-231	breast	0.82 (0.02)		1.17 (0.78)		22.02 (2.43)	
PANC1	pancreatic	1.514 (0.6503)		1.69 (0.74)		24.87 (5.13)	
ASPC1	pancreatic	14.28 (3.50)		8.16 (6.04)		>30 (0)	

Figure 5. *In vitro* response of cells to the S and R enantiomer of YK-4-279 taken from a previous publication by Dr. Uren: Oncotarget 2012; 3: 172-182**.** The racemic compound has 8.7 fold (8.88 uM/1.02 uM) therapeutic window (comparing IC50 of Ewing cells to non-Ewing Cells). The S enantiomer has 20.2 fold (6.86 uM / 0.34 uM) therapeutic window. This suggests the S enantiomer may be less toxic *in vivo*.

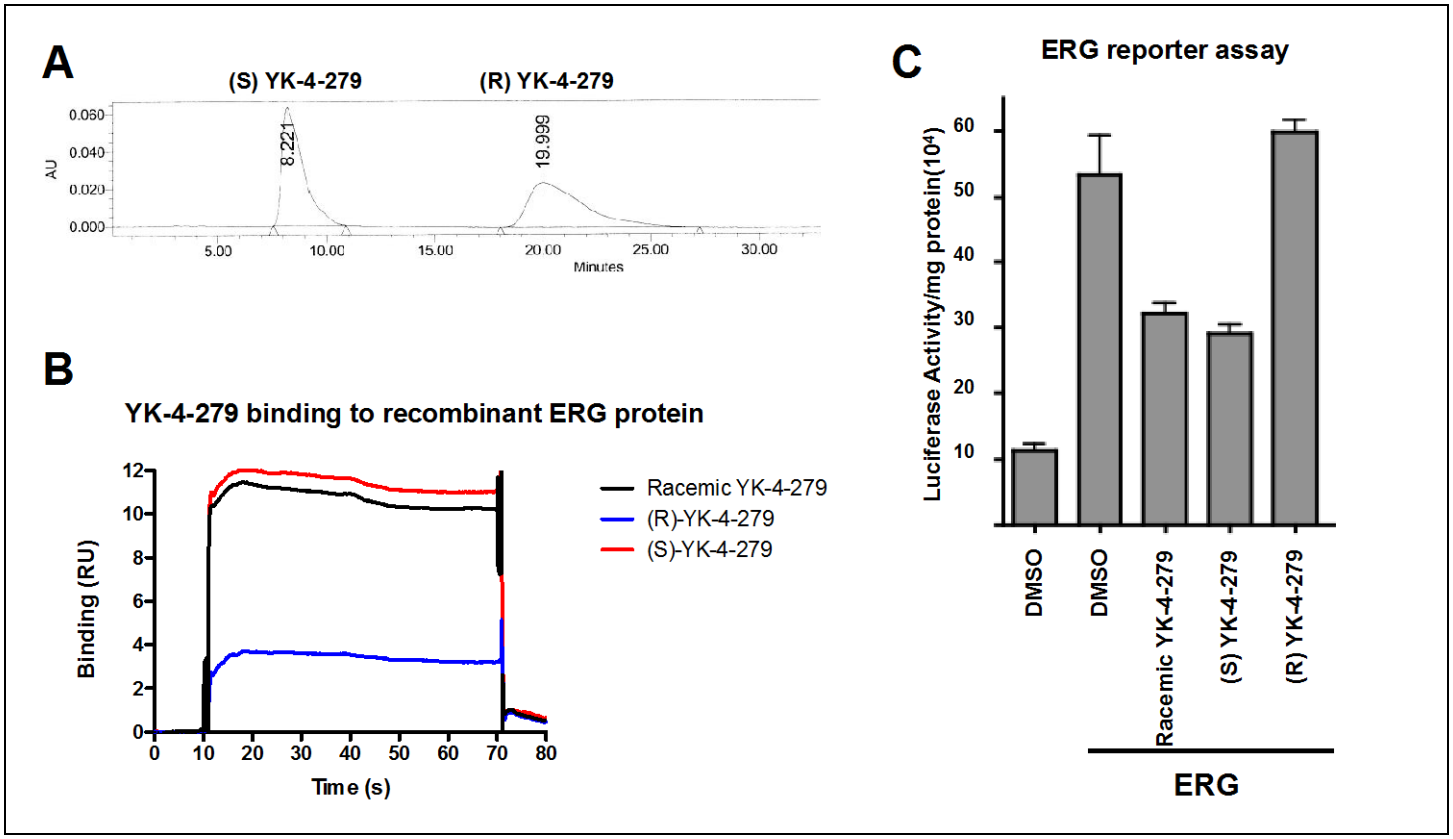


Figure 6. In the original grant Dr. Uren identified that the YK-4-279 effect is enantiomer specific. (**A**) Racemic YK-4-279 was separated to its two enantiomers by HPLC. (**B**) Recombinant ERG protein was immobilized on a Biacore CM5 sensorchip. Racemic YK-4-279 and its S and R enantiomers were injected over the protein surface to measure direct binding. (**C**) Cos-7 cells were transfected with ERG responsive luciferase reporter and an ERG expressing vector. S enantiomer that showed direct binding in Biacore also inhibited luciferase assay.

	Number of animals	Number of deaths	Mortality Rate
400 mg/kg YK-4-279	5	4	80 %
400 mg/kg (S)-YK-4-279	7	2	26 %
400 mg/kg (R)-YK-4-279	4	2	50 %

Figure 7. Two week survival data from healthy mice (5-6 week old C57BL/6J). Animals received 400 mg/kg 5 days a week for 2 weeks.

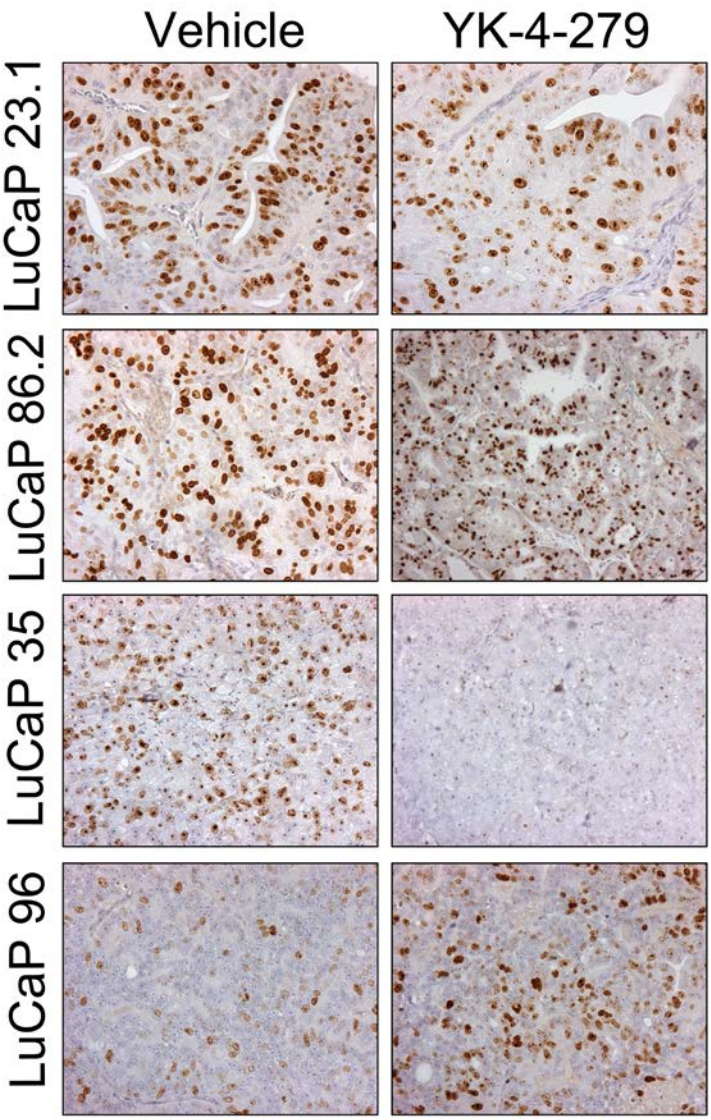
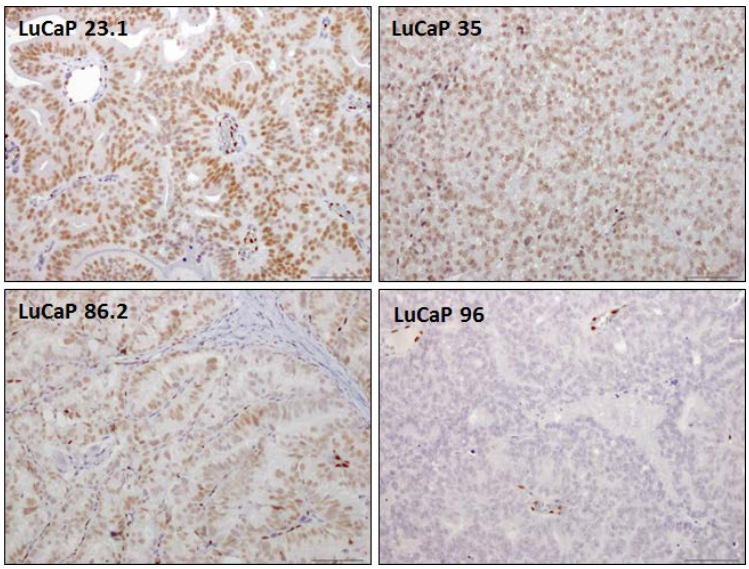


Figure 8. Ki67 staining of representative samples of the LuCaP xenografts treated with YK-4-279. LuCaP 23.1, 86.2, and 35 are all ERG positive, LuCaP 96 is ERG negative.

A



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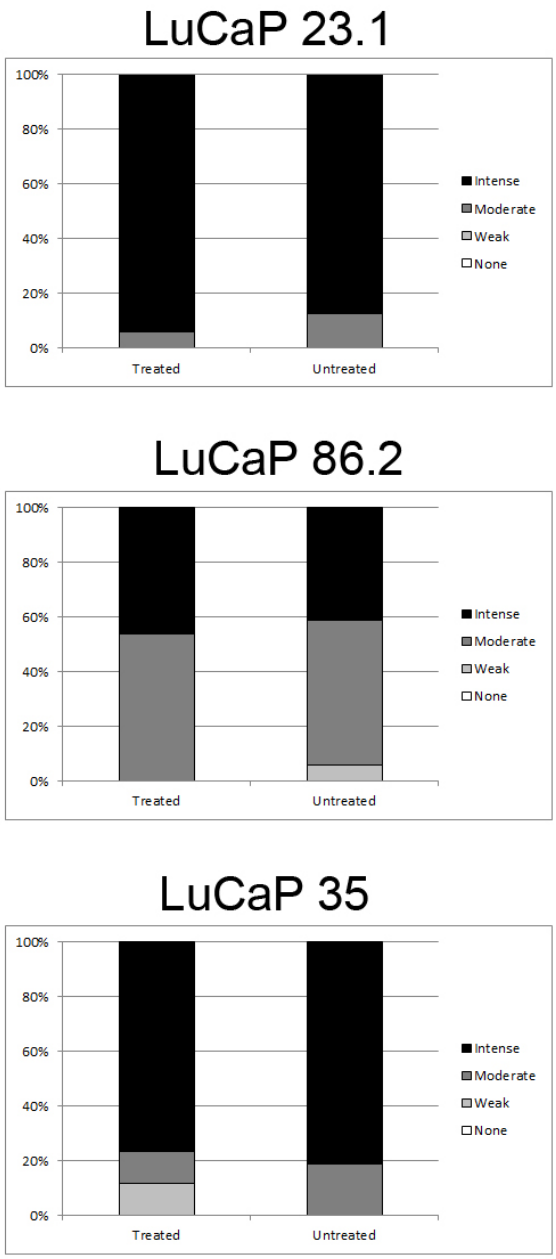

Figure 9. (A) ERG staining of representative samples of the LuCaP xenografts treated with vehicle. LuCaP 23.1, 86.2, and 35 are all ERG positive, LuCaP 96 is ERG negative. Note in LuCaP 96 there are still ERG positive endothelial cells. (B) ERG expression in YK-4-279 treated and untreated LuCaP xenografts. No significant changes in ERG expression were observed between groups.

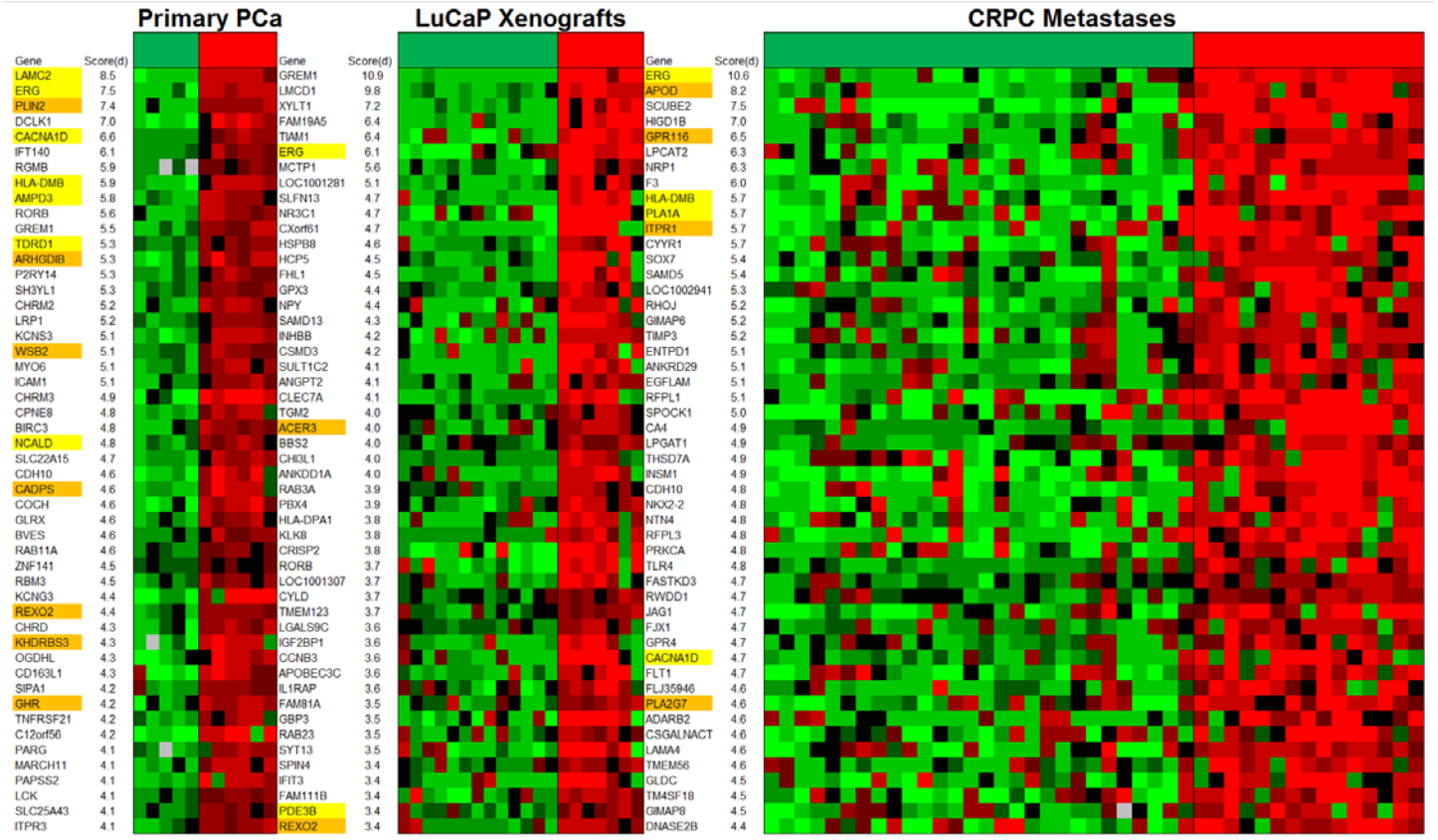

Figure 10. Genes expressed in primary prostate cancer (PCa), LuCaP xenografts and castration resistant prostate cancer (CRPC) in ERG positive (based on immunohistochemistry) vs ERG negative samples. Yellow highlights genes identified in the literature as being expressed in ERG positive tumors. Orange highlights additional genes in the literature that are identified as being expressed in ERG positive tumors by gene expression analysis but have not been validated. Note the limited number of genes differentially expressed in the LuCaP xenografts.

Key Research Accomplishments

1. The ERG inhibitor has an impact on tumor volume in patient derived xenograft models of prostate cancer.
2. Not all ERG positive tumors respond to therapy.
3. The effects of the ERG inhibitors are not non-specific as no effect was observed in an ERG negative xenograft line.
4. ERG regulated genes in prostate cancer metastases may be different to ERG regulated genes in primary prostate cancer.
5. The S-enantiomer of YK-4-279 is the active component and may be less toxic than the racemic mixture *in vivo*.

Conclusion

This data suggests ERG inhibitors can impact tumor volume in some but not all ERG positive prostate cancer tumors. It also reveals that the YK-4-279 racemic mixture has some toxicity which limits its efficacy in these studies. We will examine the S-enantiomer to determine if it will have significantly less toxicity and improve outcomes. Additionally we will determine if the decrease in tumor volume and serum PSA is due to a decrease in proliferation. Furthermore, we will use RNASeq data and immunohistochemistry to identify ERG regulated genes impacted by ERG inhibition and attempt to identify mechanisms of resistance to ERG inhibition.

Publications, Abstracts, and Presentations

This work has been presented in poster format at the PCF and AACR.

Identifying common molecular features of ERG positive tumors in primary and castration resistant prostate cancer. Colm Morrissey, Martine Roudier, Ilsa Coleman, Xiaotun Zhang, Hung-Ming Lam, Roger Coleman, Lisly Chéry, Celestia Higano, Lawrence D. True, Paul H. Lange, Eva Corey, Shiv Srivastava, Aykut Üren, Linda Snyder, Robert L. Vessella, Peter S. Nelson [Abstract of a poster presentation at the Prostate Cancer Foundation, Washington DC. October 2013].

YK-4-279 is a small molecule inhibitor of ETV1 and inhibits metastasis in a mouse model. Said Rahim, Sarah Justvig, Sung-Hyeok Hong, Perrer Tosso, Haydar Celik, Yasemin Sayedigar-Kont, Milton Brown, Colm Morrissey, Jeffrey Toretsky, Aykut Üren. [Abstract of a poster presentation at the American Association of Cancer Research, San Diego CA. April 2014].

Reportable Outcomes

This work so far has shown that ERG inhibition can result in a decrease in tumor growth, but that not all ERG positive tumors will respond to ERG inhibition.

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